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Double Heterozygosity for a *RET* Substitution Interfering with Splicing and an *EDNRB* Missense Mutation in Hirschsprung Disease

To the Editor:

Hirschsprung disease (HSCR [MIM 142623]) is a developmental disorder resulting from the arrest of the craniocaudal migration of enteric neurons from the neural crest along gastrointestinal segments of variable length (Behrman 1992). The involvement of the Ret proto-oncogene and the endothelin-B receptor-mediated signaling pathways in the migration and differentiation of enteric ganglion cells has been demonstrated in both humans and mice.

Heterozygous, incompletely penetrant point mutations and deletions of the *RET* proto-oncogene have been described in sporadic and familial cases of HSCR (Edery et al. 1994; Romeo et al. 1994; Angrist et al. 1995). In addition, homozygous *RET*-targeted disruption in mice results in megacolon with renal abnormalities (Schuchardt et al. 1994). This phenotype is reminiscent of the knockout for *GDNF*, encoding for the glial cell-line-derived neurotrophic growth factor (Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996), a protein that has been demonstrated to bind specifically and to activate Ret with a glycosylphosphatidylinositol (GPI)-anchored protein, the GDNF receptor- α (*GFRA1*) (Jing et al. 1996; Treanor et al. 1996). Although no nucleotide changes have been found at the *GFRA1* locus (Angrist et al. 1998; Myers et al. 1998), rare heterozygous mutations of the *GDNF* gene, in some cases in combination

with *RET* mutations, have been detected in a few patients (Angrist et al. 1996; Ivanchuk et al. 1996; Salomon et al. 1996). Similarly, a missense mutation in the neurturin (*NTN*) gene, a component of a second *RET* binding complex including the *NTN* receptor (also known as *GDNFR- α 2*, *GDNFR- β* , or *GFRA2*), has been found in an HSCR family cosegregating with a *RET* missense mutation (Doray et al. 1998). Rarely, megacolon presents in association with pigmentary anomalies in the Shah-Waardenburg syndrome, a human autosomal recessive disease similar to the mouse *piebald lethal* and *lethal spotting* spontaneous mutants. The study of these models of syndromic megacolon has led to the identification of homozygous mutations in the endothelin-B receptor gene (*EDNRB*) and the endothelin 3 (*EDN3*) gene in both humans and mice (Baynash et al. 1994; Hosoda et al. 1994; Puffenberger et al. 1994; Attié et al. 1995). In particular, in a large Mennonite pedigree, a founder homozygous W276C *EDNRB* mutation has been found in association with a specific *RET* haplotype, thus suggesting a possible genetic interaction between the two genes in the Mennonites affected with the Shah-Waardenburg syndrome (Puffenberger et al. 1994). *EDNRB* and *EDN3*, respectively, encode the endothelin-B receptor, a G-protein-coupled receptor with seven TM domains, and its ligand endothelin-3. We and others have demonstrated heterozygous incompletely penetrant mutations in the *EDNRB* gene in individuals with isolated megacolon (Amiel et al. 1996; Auricchio et al. 1996; Kusafuka et al. 1996), although mutations at the *EDN3* locus are rarely found in HSCR (Bidaud et al. 1997). Recently, mutations of the transcriptional factor *SOX10* (Pingault et al. 1998) and of the endothelin-

Table 1

***RET* and *EDNRB* Mutations in 50 Male Patients with Isolated HSCR**

Patient Number	Gene ^a	Exon	Nucleotide Change	Amino Acid Change	Type of Case	Length of Aganglionosis
HSCR20	<i>EDNRB</i>	4	G1151A	S305N	Sporadic	Short
HSCR18	<i>EDNRB</i>	6	del1369A	N378I	Familial	Short
HSCR46	<i>RET</i>	10	C1876A	Q626K	Sporadic	Ultrashort
HSCR20	<i>RET</i>	11	C1941T	I647I	Sporadic	Short
HSCR25	<i>RET</i>	11	G1947A	S649S	Sporadic	Short
HSCR40	<i>RET</i>	14	G2438A	R813Q	Sporadic	Short
HSCR01	<i>RET</i>	14	G2607+5A		Sporadic	Long

^a For the *RET*, *EDNRB*, and *EDN3* genes, primer sequences and PCR-SSCP conditions have been described elsewhere (Ceccherini et al. 1994; Auricchio et al. 1996). To screen the *GDNF* gene we used both SSCP (for exon 1) and denaturing gradient gel electrophoresis (DGGE) (for exon 2) with the following primer sets: 1F/R (5'-AGGCTTAACGTGCATTCTG-3' and 5'-GGGAACGGTTCTTACAGT-3'), 2AF/R (5'-30-bp GC clamp-GATCATTTTTGTCTCATG-TGCCA-3' and 5'-TCCTCTAATTCTCTGGGT-3'), 2BF/R (5'-GAGCGGAATCGGCAGGCTG-3' and 5'-30-bp GC clamp-CAAGAGCCGCTGCAGTACCT-3') and 2CF/R (5'-CTTGGGTCTGGGCTATGAA-3' and 5'-30-bp GC clamp-GCAATACACAGCAGTCTCTG-3'). PCR annealing temperatures were 57°C–63°C. SSCP was done as reported elsewhere (Ceccherini et al. 1994), whereas a 20%–70% denaturing gradient in 1 × TEA buffer at 160 V constant for 3.5–4.5 h was applied for DGGE analysis.

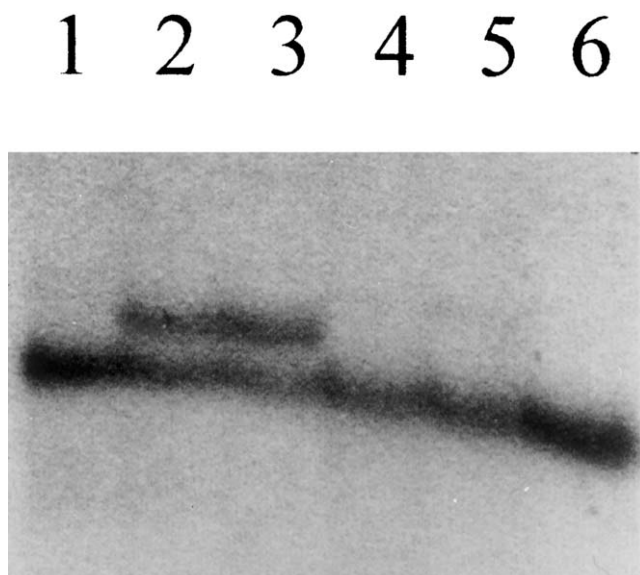


Figure 1 SSCP analysis of the *RET* exon 11 transcript from affected individuals carrying the I647I silent mutation. PCR products corresponding to this exon from father, mother, and patient 1 genomic DNA are represented in lanes 1, 2, and 3, whereas lanes 4, 5, and 6 show the PCR products obtained from the cDNA of patients 1 and 2 and a control individual, respectively. Note that the shifted upper band corresponding to the mutated allele present in lanes 2 and 3 is absent in the cDNAs of patients 1 and 2 (lanes 4 and 5), which show only the normal allele present in the cDNA from a control individual (lane 6).

converting enzyme 1 (*ECE1*) (Hofstra et al. 1998) have been reported in HSCR patients with pigmentary and cardiac defects.

RET, *GDNF*, *EDNRB*, and *EDN3* are mutated in a variable proportion of individuals affected with isolated HSCR, accounting for 30%–50% of all cases (Edery et al. 1997; Eng and Mulligan 1997; Hofstra et al. 1997). In addition, these mutations alone do not explain the variable expressivity and the incomplete penetrance of the disease for which, according to a complex model of inheritance, the combined presence of mutations in more than one of the known or still unknown HSCR susceptibility genes can be inferred.

To better understand the contribution of *RET*, *GDNF*, *EDNRB*, and *EDN3* to the pathogenesis of HSCR, we collected samples from 50 patients and analyzed them for mutations at these loci. Forty-seven of these patients represented unrelated sporadic cases, and three belonged to two different families in which a polygenic or recessive mode of inheritance could be inferred on the basis of the pedigree. They were affected with isolated megacolon, including long, short ("classic"), and ultrashort forms. None of them was born of consanguineous parents, and all were of Italian origin.

Table 1 shows two heterozygous incompletely pene-

trant *EDNRB* mutations (S305N and N378I) we recently described in two patients with isolated short-segment HSCR disease, inherited from healthy parents (Auricchio et al. 1996). These two mutations were identified during a first screening of 20 patients and were absent in 100 control chromosomes. We then analyzed samples from 30 additional patients with isolated HSCR and could not detect any novel mutations at the *EDNRB* locus. The mutation screening of the 21 exons of the *RET* proto-oncogene was done on all 50 patients. As shown in table 1, two missense mutations in exons 10 and 14 (C1876A and G2438A, respectively) and a nucleotide change affecting the 5' end of intron 14 (G2607+5A) were identified in three different patients. In particular, the Q626K missense mutation affects the last codon of exon 10, namely the cys-rich region located in the juxtamembranous extracellular domain, suggesting either an effect on the folding of the ligand-binding domain or an interference with *RET* dimerization, a crucial step preceding its activation. Mutation R813Q, affecting exon 14, is likely to alter the *RET* tyrosine kinase activity, whereas the G→A transition at the 5' end of intron 14 might interfere with correct processing of the primary transcript. In addition, we found two synonymous nucleotide changes in *RET* exon 11, leading to neutral substitutions I647I and S649S in patients 20 and 25, respectively (table 1). These were absent in 150 control individuals. We could not detect any *GDNF* or *EDN3* mutations in our patients, thus confirming that they play a minor role in HSCR pathogenesis.

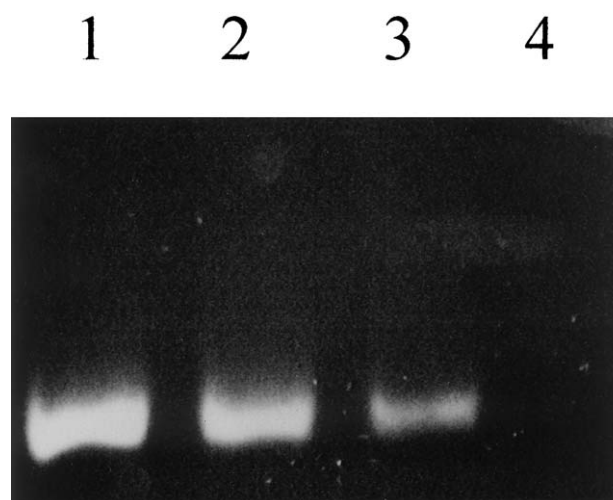


Figure 2 ARMS analysis of the *RET* exon 11 from patient 2 genomic DNA and cDNA. PCR products were obtained by use of oligonucleotide primers specific for either the normal (lanes 1 and 3) or the I647I (lanes 2 and 4) *RET* alleles. A band of the expected size is amplified from patient genomic DNA with both sets of primers (lanes 1 and 2), whereas at the cDNA level the normal (lane 3), but not the mutated, allele (lane 4) is present.

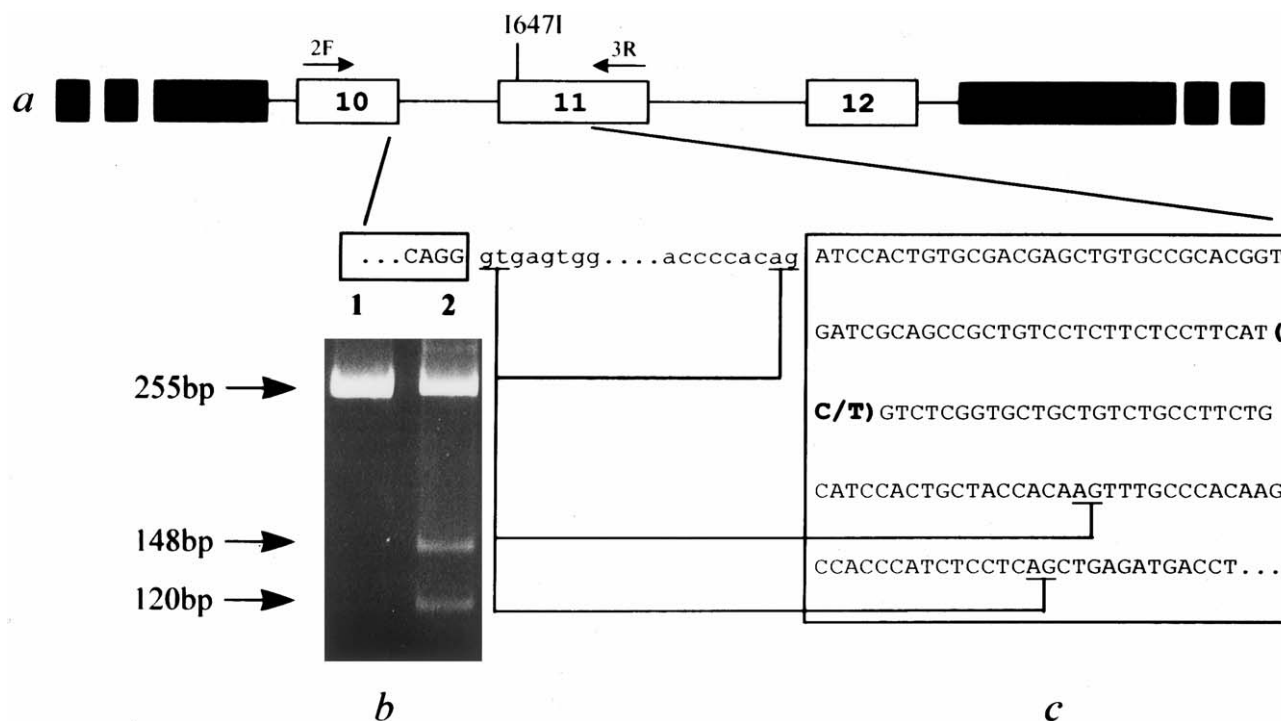


Figure 3 In vitro analysis of the *RET* exons 10 and 11 splicing products from both the normal and the I647I mutated alleles (see text for details). *a*, Schematic representation of the *RET* constructs used for the in vitro splicing analysis. *b*, RT-PCR amplification using primers designed in exons 10 and 11, as indicated in *a*, from total RNA of COS-7 cells transiently transfected with the *RET* wild-type (lane 1) and the I647I (lane 2) constructs. Note the abnormal products present in lane 2. *c*, Sequence analysis of the 255-bp products from lanes 1 and 2 shown in *b* corresponded to the normal and mutated *RET* alleles, respectively, an observation consistent with the use of canonical "gt" and "ag" donor and acceptor splice sites (underlined in the partial intronic sequence given in lowercase letters). Conversely, sequence analysis of the 148-bp and 120-bp products revealed two exon 11 deleted forms resulting from the use of cryptic acceptor "ag" sites activated to generate the two abnormal products (underlined in the sequence of the 5' portion of exon 11 given in capital letters). The two alternative nucleotides involved in the neutral substitution of codon 647 (T in the normal allele and C in the variant allele) are shown in boldface type.

Interestingly, patient 20 (from now on, patient 1) carries both a *RET* synonymous nucleotide change, C1941T (I647I), inherited from the unaffected mother, and an *EDNRB* missense mutation, S305N, transmitted by the healthy father. This latter mutation results in the substitution of a serine, located in the third intracellular loop, with an asparagine (Auricchio et al. 1996). This serine, highly conserved between different species, has been shown to be a site of in vivo phosphorylation (Roos et al. 1998), suggesting that it plays an important role in the receptor regulation and that its absence can result in the protein loss of function. Intraexonic silent mutations can alter correct mRNA processing, thus resulting in either altered mRNA levels or truncated proteins (Steingrimsdottir et al. 1992; De Meirleir et al. 1994; Li et al. 1995; Richard and Beckmann 1995; Jin et al. 1996; Llewellyn et al. 1996; Ploos van Amstel et al. 1996; Liu et al. 1997). To test the hypothesis that the *RET* I647I variant could play a causative role in the development of the disease phenotype in patient 1, we analyzed, both in vitro and in vivo, the *RET* mRNA expression, using

cDNA and genomic DNA from patient 1 and from an additional proband (patient 2) with the same I647I change, described elsewhere (Ceccherini et al. 1994).

As an in vivo approach to study the *RET* transcription, we performed reverse transcription (RT) PCR with total RNA from lymphoblastoid cell lines of these two patients, by means of two different techniques—the SSCP (Orita et al. 1989) and the amplification-refractory mutation system (ARMS) (Newton et al. 1989) analysis. Results obtained from SSCP analysis of the *RET* exon 11 are shown in figure 1. The shifted upper band corresponding to the mutated allele present in the genomic DNA of patient 1 and his healthy mother (lanes 2 and 3) is absent in the cDNAs of patients 1 and 2 (lanes 4 and 5). They depict only the normal allele present in the cDNA from a control individual (lane 6). Similar results have been obtained from ARMS analysis of the *RET* exon 11 from patient 2 genomic DNA and cDNA (fig. 2). In particular, using oligonucleotide primers specific for either the normal (lanes 1 and 3) or the I647I (lanes 2 and 4) *RET* alleles, we were able to amplify a band

of the expected size from patient genomic DNA with both sets of primers (lanes 1 and 2), although at the cDNA level the normal (lane 3), but not the mutated, allele (lane 4) was present. In addition, direct sequence analysis of the PCR product, as well as *BsmI* enzymatic restriction of an exon 7 *RET* polymorphism, the phase of which is known with respect to the I647I mutation, confirmed the absence of the mutated allele in the cDNA of the affected individuals (data not shown).

To elucidate whether the I647I silent mutation could interfere with splicing, thus resulting in unstable products undetectable in vivo in lymphoblast cDNAs, we cloned the 3-kb genomic region encompassing *RET* exons 10, 11, and 12, from both the normal and the I647I mutated alleles, into the pSPL3 eukaryotic vector (Church et al. 1994) (fig. 3a). RT-PCR amplification from total RNA of SV40-transformed African green monkey kidney cells (COS-7) transiently transfected with the *RET* wild-type construct (fig. 3b) shows the expected fragment of 255 bp (lane 1). Two additional products of 148 bp and 120 bp were amplified when the I647I construct was transfected (lane 2). As shown in figure 3c, sequence analysis of the 255-bp products from the above lanes 1 and 2 corresponded to the normal and mutated *RET* alleles, respectively, an observation consistent with the use of canonical "gt" and "ag" donor and acceptor splice sites (underlined in the partial intronic sequence given in lowercase letters). Conversely, sequence analysis of the 148-bp and 120-bp products revealed two exon 11 deleted forms. Both products resulted from the fusion of the 3' end of exon 10 to two different sites located 107 bp and 135 bp downstream of the exon 11 starting nucleotide in the largest and the smallest abnormal products, respectively. If translated, these mRNAs would result in two proteins: the one corresponding to the largest transcript truncated 758 bp downstream of the newly activated splice site, and the smallest mRNA resulting in an in-frame interstitial loss of 45 amino acids, including the transmembrane domain. Nevertheless, we postulate that this abnormal processing of the I647I allele may reduce the mutant RNA to a level that escapes detection by the commonly used in vivo RT-PCR approach. Thus, the presence in our patient of an inactive form of the endothelin-B receptor and of reduced levels of the Ret protein could impair the normal enteric neuronal migration.

In conclusion, we confirm that in isolated HSCR the major susceptibility locus is the *RET* proto-oncogene, with *EDNRB* accounting for a minority of cases. More relevantly, we demonstrate in two different patients, both in vivo and in vitro, that the same silent *RET* mutation can interfere with correct transcription, thus possibly leading to a reduced level of the Ret protein. Finally, the coexistence, reported for the first time, in the same patient of two functionally significant *EDNRB* and *RET*

mutations suggests a direct genetic interaction between these two distinct transmembrane receptors in polygenic HSCR disease.

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Electronic-Database Information

Accession number and URL for data in this study are as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (for HSCR [142623])

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A Mutation (2314delG) in the Usher Syndrome Type IIA Gene: High Prevalence and Phenotypic Variation

To the Editor:

Usher syndrome (USH), an autosomal recessive condition, is characterized by hearing impairment associated with retinitis pigmentosa (RP). It is a clinically and genetically heterogeneous condition. Three clinical forms of USH have been described, and eight loci have been mapped (Hereditary Hearing Loss home page). USH type I (USH1) is the most severe form, manifested by profound congenital deafness, constant vestibular dysfunction, and prepubertal onset of RP. USH type II (USH2) is characterized by congenital moderate-to-severe hearing impairment, normal vestibular responses, and RP (Smith et al. 1994). USH type III (USH3) is characterized by progressive hearing loss, variable vestibular problems, and RP (Pakarinen et al. 1995). The *USH1B* gene has been identified (Weil et al. 1995), and a variety of mutations leading to USH1 have been catalogued in the *MYO7A* gene (Liu et al. 1998). USH2 is

the most common of the three types of USH, accounting for more than half of all cases (Hopes et al. 1997; Rosenberg et al. 1997).

Recently, Eudy et al. (1998) reported the identification of the USH type IIA gene (*USH2A*; MIM 276901). *USH2A* encodes a putative extracellular matrix protein of 1,551 amino acids with laminin epidermal growth factor and fibronectin type II motifs. Expression of the *USH2A* gene has been detected by reverse transcriptase PCR (RT-PCR) from fetal human cochlea, eye, and adult human retina (Eudy et al. 1998). Three different mutations were identified in patients with *USH2A*, the most frequent of which is the 2314delG mutation. Of 96 probands tested, 21 carried the 2314delG mutation.

In the present study, we have undertaken mutational analysis of the *USH2A* gene in 23 families with USH (both USH2 and atypical USH), from the United Kingdom and China. We found that the majority of families with USH2 carry the 2314delG mutation. Surprisingly, we determined that the 2314delG mutation in the *USH2A* gene can also lead to atypical USH.

Of 23 families with USH analyzed in this study, 15 have been described elsewhere (Hopes et al. 1997). All available affected subjects were examined by one of the authors. Full clinical histories were obtained, with emphasis on any potential causes of hearing impairment. The audiovestibular and ophthalmic evaluations were done on the basis of recommendations by the Usher Syndrome Consortium (Smith et al. 1994). The details of the clinical evaluations can be found in Hopes et al. (1997). The cases studied here are classified clinically into the following two groups: (1) USH2, consisting of congenital sloping moderate/severe hearing impairment, normal vestibular function, and RP (Smith et al. 1994); and (2) atypical USH, consisting of bilateral sensorineural progressive hearing loss without other obvious factors as the cause of the progression of the hearing impairment, along with variable vestibular dysfunction and RP.

Of 23 families studied, 13 were given a diagnosis of USH2 and 10 were given a diagnosis of atypical USH. Eighteen families living in the United Kingdom and five in China were included in our analyses. Twenty-three probands with USH were screened for the 2314delG mutation by combined SSCP/heteroduplex analysis. Twelve families (52%) carried the 2314delG mutation—8 (62%) of 13 patients with typical USH2 and 4 (40%) of 10 patients with atypical USH (table 1). Of 12 families with this mutation, 7 were identified with only one affected member (sporadic cases). Of the remaining five families with more than one affected individual, two families (USH.05 and USH.10) with two affected sibs were tested for cosegregation of the disease with the *USH2A* locus (Eudy et al. 1998). The segregation analysis was consistent with linkage to *USH2A* (data not